Expression of human cardiac-specific genes: a novel method for post-mortem interval estimation

Ekspresja genów specyficznych dla ludzkiego serca: nowa metoda szacowania interwału pośmiertnego

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Abstract
In legal medicine, the determination of post-mortem interval (PMI) is not only an important but also one of the most difficult aspects. Several methods are used to estimate PMI such as physicochemical, entomological, biochemical, metabolic, autolytic, and physical methods. These methods provide a wide range of PMI as they are affected by different factors. The approach behind the present study is to calculate an accurate PMI by using mRNA degradation and fold change expression (FCE) of cardiac-specific genes viz. N-terminal pro-B-type natriuretic peptide (NPPB) and cardiac troponin I (TNNI3). Seventeen cadaver heart tissues were analysed within a time frame of up to 12 hours from the time since death, at different time intervals at room temperature. Gene expression was determined and the data were analysed using the value of average delta Ct (ΔCt) value of the assessed gene and housekeeping gene. Delta delta Ct (ΔΔCt) method was used to calculate the FCE at the different 7-time groups. The FCE of TNNI3 was almost stable till 15 hours of PMI and then after 15 hours, expression shows a decrease up to 24 hours after death; whereas, NPPB shows that FCE was stable till 12 hours of PMI and then after 12 hours, expression shows a decrease up to 24 hours after death. The FCE of NPPB and TNNI3 was almost stable till 12 hours. Thus, the estimation of PMI by analysis of the FCE of cardiac-specific genes can be a new promising method in forensic medicine.

Keywords
cardiac troponin I, fold change expression, forensic genetics, GAPDH, N-terminal pro-B-type natriuretic peptide, post-mortem interval.

Streszczenie

W medycynie sądowej określenie interwału pośmiertnego (PMI) jest nie tylko ważnym, ale i jednym z najtrudniejszych aspektów. Do oszacowania PMI stosuje się kilka metod, takich jak metody fizykochemiczne, entomologiczne, biochemiczne, metaboliczne, autolityczne i fizyczne. Metody te zapewniają szeroki zakres PMI, ponieważ wpływają na nie różne czynniki. Podejście zastosowane w niniejszym badaniu polega na obliczeniu dokładnego PMI przy użyciu degradacji mRNA i zmiany krotności ekspresji (FCE) genów specyficznych dla serca, a mianowicie N-końcowego peptydu natriuretycznego typu pro-B (NPPB) i troponiny sercowej I (TNNI3). Siedemnaście tkanek serca ze zwłok analizowano w przedziale czasowym do 12 godzin od czasu śmierci, w różnych odstępach czasu w temperaturze pokojowej. Określono ekspresję genów, a dane przeanalizowano, stosując wartość średniej wartości delta Ct (ΔCt) ocenianego genu i genu metabolizmu podstawowego. Metodę delta delta Ct (ΔΔCt) zastosowano do obliczenia FCE w różnych grupach. FCE dla TNNI3 była prawie stabilna do 15 godzin PMI, a następnie po 15 godzinach ekspresja wykazała spadek do 24 godzin po śmierci; podczas gdy FCE dla NPPB była stabilna do 12 godzin PMI, a następnie po 12 godzinach ekspresja wykazała spadek do 24 godzin po śmierci. FCE dla NPPB i TNNI3 były prawie stabilne do 12 godzin. Zatem oszacowanie PMI poprzez analizę FCE genów specyficznych dla serca może być nową obiecującą metodą w medycynie sądowej.

Słowa kluczowe

troponina sercowa I, krotność zmiany ekspresji, genetyka sądowa, GAPDH, N-końcowy pro-B-typowy peptyd natriuretyczny, interwał pośmiertny

Introduction

Estimating the time since death is one of the most important and challenging aspects of forensic medicine. In legal medicine, the accurate determination of the time of death is important due to its role in elucidating possible criminal acts and determining appropriate civil repercussions. Since time immemorial, it has been attempted to estimate post-mortem interval (PMI) using various techniques. Previous methods of estimating PMI included physicochemical, entomological, biochemical, metabolic, autolytic, and physical methods. The latter, such as rigor mortis, post mortem staining, algor mortis, and putrefaction offer a broad range of time since death as they are affected by various factors (1). However, these were never accurate, and the quest for better methods led to the development of post-mortem genetics, including the assessment of mRNA degradation and gene expression. Molecular biology is a new area on which researchers are concentrating at present. Various studies used different tissues as source mRNA and found a clear relationship between PMI and gene expression of different biomarkers. RNA stability has been described in the retina (2), human bone (3), foetal and neonatal lung tissues (4), blood (5,6), etc. The N-terminal pro-B type natriuretic peptide (NPPB) and cardiac troponin I (TNNI3) are the leading cardiac biomarkers as per currently available literature. Numerous studies have been conducted to assess the level of cardiac biomarker proteins in different post-mortem samples such as antemortem and post-mortem serum levels, pericardial fluids, cerebrospinal fluids, and the non-human heart (7,8). In the present study, human heart cadaver tissue has been used and the approach behind it is to assess the fold-change gene expressions (FCE) of the cardiac muscle-specific genes at different time intervals (0,6,12 hours) to calculate the time since death. The data was analyzed using the delta delta ct (ΔΔCt) value to calculate the FCE at the different 7-time groups (0-6 hrs, 6-9 hrs, 9-12 hrs, 12-15 hrs,15-18 hrs,18-21 hrs, 21-24 hrs). Livak's method was used for the FCE analysis, whereas GAPDH was used for the normalization of target gene expression (9,10).

Material and Methods

Location, study design, and cadaver cases

The present study was approved by the Institutional Ethics Committee and was conducted on the South Asian population aged between 15-55 years with the BMI value ranging from 16.5 to 26 kg/m^2 between the years 2020-2021 using seventeen male cadaver heart tissues within a time frame of up to 12 hours since death. The study was performed at the Department of Forensic Medicine and Biochemistry and followed the principles of the Declaration of Helsinki for medical research. Before enrolling each deceased into the study, a written informed consent was taken from family members. All medico-legal autopsies were included in which the time since death was known, no previous history of cardiac illness was present, and no gross cardiac abnormality was detected at the time of autopsy. Only the cases in which the autopsy was performed within 12 hours after death were included. The cases of death due to chronic obstructive pulmonary disease with cor-pulmonale or respiratory failure, acute pulmonary embolism, hyperthyroidism, acute or chronic kidney injury, sepsis, pulmonary hypertension, and cardiac disease were excluded to avoid the confounding effect of the abovementioned pathologies on cardiac-specific genes.

Study Scheme

The samples were collected at the time of autopsy performed within 12 hours of death. The time of death was assumed as time point zero, in such a way that the first sample taken, fell within one of the time intervals (0, 6, 12 hr from the time of death). The next samples were taken 6 and 12 hours from the time of the first sampling, respectively. The corpses were in-hospital deaths, however, not all of them were received in the mortuary within the stipulated time due to logistic issues, hence the time period that elapsed between the time of death and the first sampling varied between the cases. Therefore, the samples were grouped together in time intervals to bring uniformity to the data and more accurate analysis, barring the bias created by different time points. Thus, we analyzed 3 genes at 3 different time points, and consequently, we obtained 9 samples (3×3) per cadaver, whereas for 17 cadavers we analysed a total of 153 tissue samples (17×9).

Cadaver sample and homogenization

The dissection of the heart was performed using a sterile scalpel, scissors, forceps, and thus 10g of right ventricular heart tissue was harvested. After collection, within 5 minutes, the tissue was transported in a universal container holding chilled phosphate-buffered saline (PBS) to the biochemistry laboratory for further processing.

Sample processing and RNA isolation

The samples were immediately processed for nucleic acid extraction. A 100 mg cardiac tissue sample was taken and RNA was isolated. This was labelled as the first sample. Thereafter, 100 mg cardiac tissue samples were harvested 6 and 12 hours after the first sample, respectively, and then processed. Total RNA was isolated using Trizol (RNA-XPress™ Reagent, HiMedia Laboratories) according to the manufacturer's instructions. The quantity and quality of isolated RNA were measured using a nanodrop reader (BioTek Instruments, Inc., Vermont, USA). Extracted RNA samples with a 260/280 and 260/230 ratio of ≥ 1.8 and ≥ 2 were considered acceptable for further processing and stored at -80°C.

cDNA conversion and quantitative real-time polymerase chain reaction

The conversion of cDNA from 500 ng of RNA of each sample of different time intervals was reverse transcribed using BIO-RAD iScript cDNA Synthesis Kit (Catalog n. 1708891) as per the manufacturer's protocol. 20 µL reaction mixture by volume was prepared and incubated in a thermal cycler following the protocol, and reverse transcription was performed at 46°C for 20 minutes. Since using the NCBI BLAST technique, all the mRNA primers for TNNI3, NPPB, and GAPDH were designed and purchased from Sigma. The primer sequence used for qRT-PCR was as follows:

Gene	Forward	Reverse
TNNI3	5'-GCAAGAAAAAGTTT-GAGAGC-3'	5'-TTTTTCAGCTCAGA-GAGAAG-3'
NPPB	5'-ATTAAGAGGAAGTC-CTGGC-3'	5'-AAATGAGTCACTTC-AAAGGC-3'
GAPDH	5'- ACAGTTGCCATG-TAGACC-3'	5'- TTGAGCACAGGG-TACTTTA-3'

The qRT-PCR amplification and expression analysis was performed using CFX96 RealTime System and CFX Manager Software (Bio-Rad, California, USA). Since using SYBR Green qPCR Kit, all qRT-PCR was performed according to the manufacturer's instructions with the use of Thermo Scientific DyNAmo Color Flash. To confirm the specific gene amplification, the melt curves (55°C-95°C) were generated and each sample was run in duplicates. To optimize the annealing temperature gradient, PCR was used for each primer. The qRT-PCR was performed under the following conditions: PCR initial activation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 56°C (for all genes) for 60 seconds. The Ct values of cDNA were normalized by using the mean cycle threshold (Ct) values of GAPDH.

Table I. Average ΔCt value of cardiac troponin I at different time groups

Groups	Group 1 (0-6 h)	Group 2 (6-9 h)	Group 3 (9-12 h)	Group 4 (12-15 h)	Group 5 (15-18 h)	Group 6 (18-21 h)	Group 7 (21-24 h)	Standard Deviation
Average ΔCt value	-1.57	-1.52	-1.45	-1.42	-1.33	-1.56	-1.79	0.146

Table II. Values of ΔΔCt and fold change expression of TNNI3 at different time groups

ΔΔCt Value ΔΔCt = ΔCt(E) – ΔCt(C)	$\Delta Ct_{(G2)} - \Delta Ct_{(G1)}$	$\Delta Ct_{(G3)} - \Delta Ct_{(G2)}$	$\Delta Ct_{(G4)} - \Delta Ct_{(G3)}$	$\Delta Ct_{(G5)} - \Delta Ct_{(G4)}$	$\Delta Ct_{(G6)} - \Delta Ct_{(G5)}$	$\Delta Ct_{(G7)} - \Delta Ct_{(G6)}$	Standard Deviation
Delta delta ct value	0.05	0.07	0.03	0.09	-0.23	-0.23	0.15
Fold Change Expression (2^-ΔΔCt)	1.03	1.04	1.02	1.06	0.85	0.85	0.09

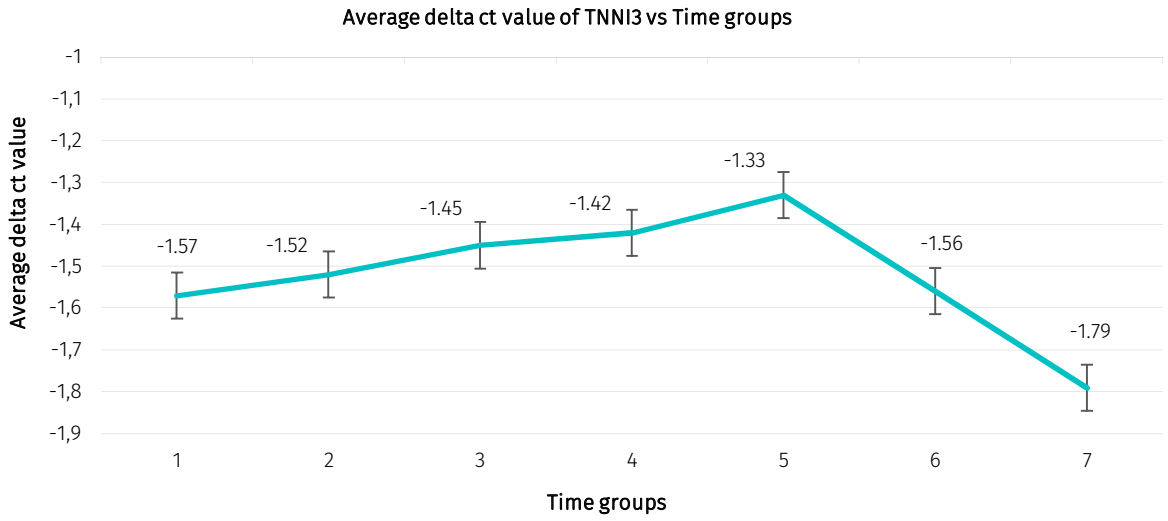

Figure I. The ΔCt value of the TNNI3 gene of the cardiac muscle at different time groups

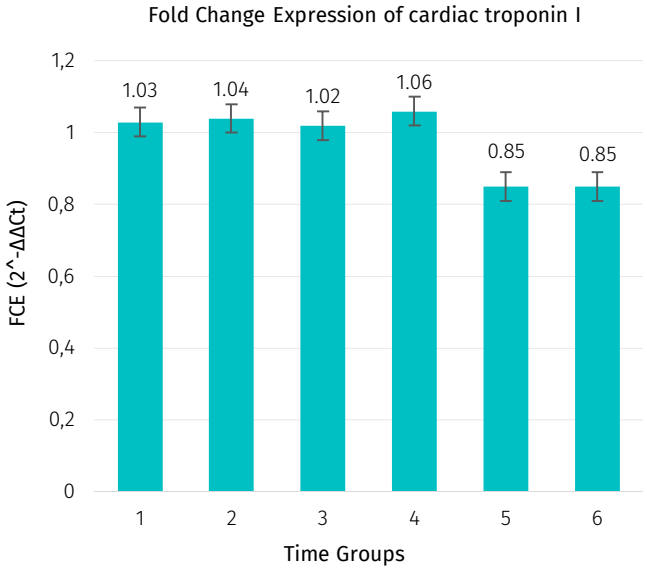

Figure II. Fold change expression by ΔΔCt of TNNI3 value with PMI

Statistical analysis

Statistical analysis was conducted by R Commander (R version 4.2.0) and evaluated employing the ΔΔCt method. The t-test for independent variables was applied to the delta Ct values to obtain the p values. Statistical significance was considered for p values <0.05.

Result

The data obtained using the abovementioned method, after the completion of qRT-PCR was expressed in the cycle threshold value (Ct). Ct value of cardiac-specific biomarkers of the assessed gene (TNNI3, NPPB) and internal housekeeping gene (GAPDH) at different time points were further analysed. The data was analyzed using the delta delta ct (ΔΔCt) value to calculate the FCE at the different 7-time groups (0-6 hrs, 6-9 hrs, 9-12 hrs, 12-15 hrs, 15-18 hrs, 18-21 hrs, 21-24 hrs).

Table III. Average ΔCt value of NPPB at different time groups.

Groups	Group 1 (0-6 h)	Group 2 (6-9 h)	Group 3 (9-12 h)	Group 4 (12-15 h)	Group 5 (15-18 h)	Group 6 (18-21 h)	Group 7 (21-24 h)	Standard deviation
Average ΔCt value	1.62	2.21	2.84	3.42	3.14	2.03	0.55	0.98

Table IV. Values of ΔΔCt and fold change expression of NPPB at different time groups

ΔΔCt Value $\Delta\Delta Ct = \Delta Ct_{(E)} - \Delta Ct_{(C)}$	$\Delta Ct_{(G2)} - \Delta Ct_{(G1)}$	$\Delta Ct_{(G3)} - \Delta Ct_{(G2)}$	$\Delta Ct_{(G4)} - \Delta Ct_{(G3)}$	$\Delta Ct_{(G5)} - \Delta Ct_{(G4)}$	$\Delta Ct_{(G6)} - \Delta Ct_{(G5)}$	$\Delta Ct_{(G7)} - \Delta Ct_{(G6)}$	Standard Deviation
Delta delta ct value	0.59	0.63	0.58	-0.28	-1.11	-1.48	0.93
Fold Change Expression (2^-ΔΔCt)	1.50	1.54	1.49	0.82	0.46	0.35	0.55

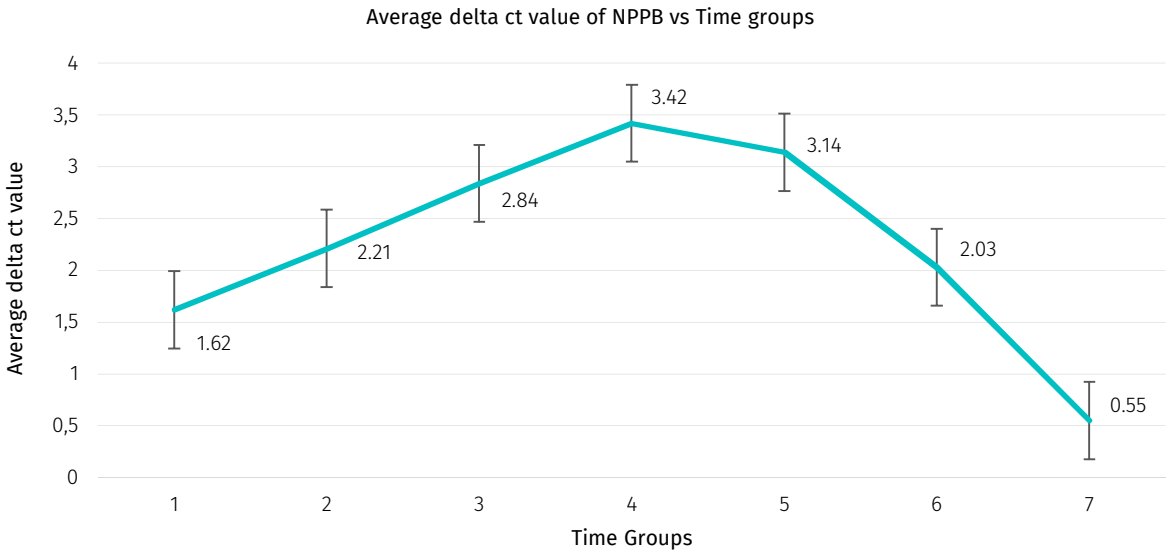

Figure III. The ΔCt value of the NPPB gene of the cardiac muscle at time interval

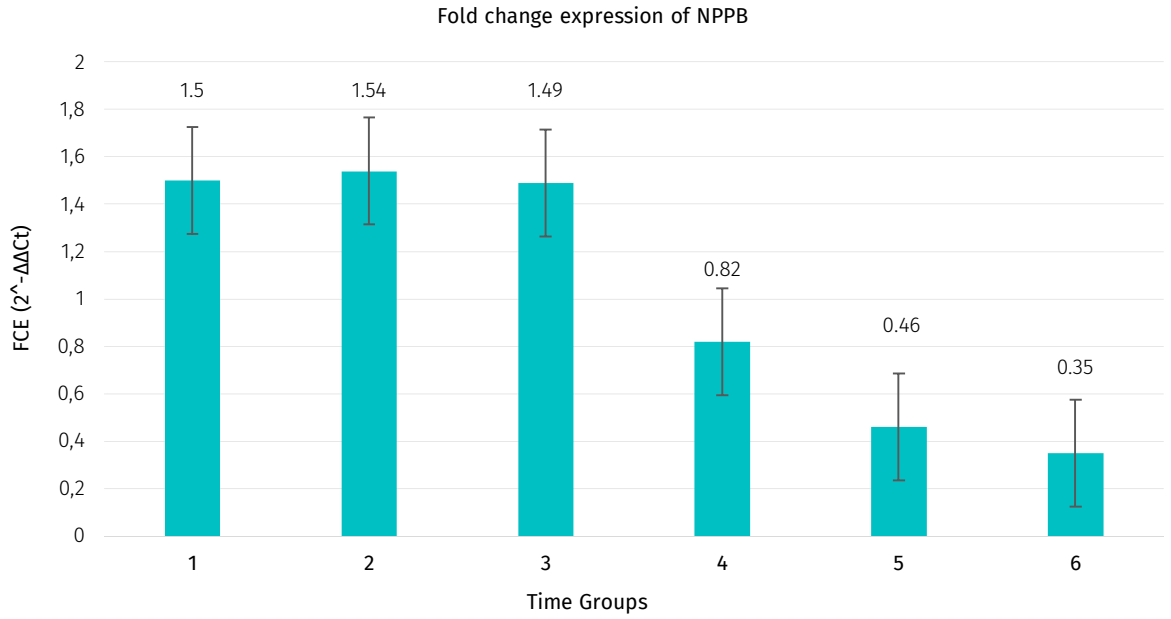

Figure IV. Fold change expression by ΔΔCt of NPPB value with PMI

ΔCt (sample) = ΔCt (assessed gene) – ΔCt (housekeeping gene)
ΔΔCt = ΔCt (experimental) – ΔCt (control)
Fold Change Expression = 2^-ΔΔCt

Cardiac troponin I

On plotting the average ΔCt value of TNNI3 graphically at different time intervals, a continuous increase in the levels of cardiac troponin I up to 15-18 hours and then a decrease up to 24 hours of PMI was observed (Figure I). The average ΔCt value of cardiac troponin I at different time groups is shown in Table I.

The fold change expression (FCE) of cardiac troponin I by ΔΔCt method at different time intervals showed that the FCE of TNNI3 was almost stable till 15 hours of PMI and then after 15 hours, it showed a decrease up to 24 hours after death. The values of delta delta ct (ΔΔCt) and FCE with PMI are shown in Table II and Figure II.

A simple sample t-test was applied to the delta Ct values of TNNI3 with GAPDH and the result was found to be significant (p-value 0.0000001518) at different time intervals.

N-terminal pro-B-type natriuretic peptide (NPPB)

The plotting of the average ΔCt value of NPPB graphically at different time intervals showed that there was a linear increase in the levels of NPPB up to 12 to 15 hours of PMI followed by a drop at 15 to 18 hours of PMI, and continuously decreased up to 24 hours after death (Figure III). The average ΔCt value of NPPB is shown in Table III below.

The FCE of NPPB by ΔΔCt method at different time intervals showed that FCE was stable till 12 hours after death and then after 12 hours of PMI, it showed the decrease up to 24 hours after death. The values of delta delta Ct (ΔΔCt) and FCE are shown in table IV and figure IV.

A simple sample t-test was applied to the delta Ct values of NPPB with GAPDH and the result was found to be significant (p-value 0.0000001518) at different time intervals.

Discussion

One of the most mysterious questions in forensic medicine is how to estimate the time since death. If it is accurately estimated, then a lot of controversies can be resolved. The objective of the present study was to determine the gene expression of cardiac-specific genes for application in PMI. Numerous studies were conducted in the past to estimate the PMI by analyzing the cardiac biomarkers, TNNI3 and NPPB. Blood serum and pericardial fluid were used as samples in these studies to compare the antemortem and post-mortem values of the abovementioned biomarkers. The analysis shows a concordant trend of post-mortem TNNI3 with PMI in sudden cardiac death up to 12 hours but no successful correlation could be drawn between the antemortem and post-mortem levels of the same (7,11,12). To the best of our knowledge, there is no reported study on human TNNI3 and ProBNP levels and their gene expression pattern for estimation of the time since death. The only reported study to estimate the PMI using protein marker cardiac troponin I was conducted on non-human (bovine) cadaver heart tissue on six samples and it showed a pseudo linear relationship between percent TNNI3 and the log of the time since death (r >0.95) (8). The ΔCt value of the TNNI3 gene of the cardiac muscle showed almost equal degradation at equal time intervals correlated with PMI within 0 to 12 hours at room temperature (13). Michaud et al. conducted a study on post-mortem NPPB measurement on 96 autopsy cases in 2007. The goal of the study was to determine the post-mortem stability of NPPB and to measure the amounts of NPPB in the heart of people who had myocardial ischemia. It highlighted the relationships between different autopsy specimens (e.g., pericardial fluid, vitreous humour, serum, and blood). The findings suggested that NPPB levels were much greater in those who had a heart attack and that there were good connections between NPPB levels in different samples of pericardial fluid, femoral blood, and serum (14). In 2016, Tettamanti et al. conducted a study on 16 forensic autopsy cases to determine the levels of NPPB, troponin I, and troponin T in sepsis-related deaths from femoral blood. The levels of serum troponin T, troponin I, and NPPB were all higher in sepsis-related mortality, according to the study (15). In 2017, Palmiere et al. published an overview of a study on post-mortem intervals utilizing cardiac troponins and NPPB. The researchers compared antemortem serum levels of these markers to post-mortem levels detected in pericardial fluid and post-mortem serum samples collected from various sampling locations. The findings showed that when measuring cardiac troponins in post-mortem samples, the time since death should always be taken into account (7). The approach behind our study was to analyze the gene expression of TNNI3 and NPPB. Our study has been by far, the only human-based study up to the present moment. The result of our study indicated that the FCE of TNNI3 was almost stable till 15 hours of PMI, thereafter the expression showed a decrease up to 24 hours after death. On the contrary, a continuous increase in the Ct values of TNNI3 was observed up to 15-18 hours followed by a decrease in Ct value up to 24 hours of PMI. The NPPB showed that FCE was stable till 12 hours after death followed by a decrease in FCE in 12-24 hours intervals after death. The linear increase in the Ct values of NPPB observed up to 12 to 15 hours of PMI was followed by a drop at 15 to 18 hours of PMI and a subsequent decrease up to 24 hours after death. The objectivity and reliability of this scientific method provide a stronger value in comparison to physical methods and, when applied properly, it can be more reliable as compared to other methods. The uniqueness of exploring such new methods lies in their accuracy and objectivity in estimating PMI.

Conclusion

The study demonstrates the result that the FCE gene expres-

sion of NPPB and TNNI3 of cadaver heart tissue is dependent on PMI, with the FCE showing a decrease in TNNI3 and NPPB, in the heart tissue with increasing time of death. Thus, the estimation of PMI by analysis of the FCE of the cardiac-specific genes is a new and promising method in forensic medicine. The uniqueness of exploring such new methods lies in their accuracy and objectivity in estimating PMI.

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